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Antibody Responses in the Lungs of Mice following Oral Immunization with *Salmonella typhimurium aroA* and Invasive *Escherichia coli* Strains Expressing the Filamentous Hemagglutinin of *Bordetella pertussis*

CARLOS A. GUZMÁN,†* ROBERT M. BROWNLIE, JAGATH KADURUGAMUWA, MARK J. WALKER,‡ AND KENNETH N. TIMMIS

Department of Microbiology, GBF—National Research Center for Biotechnology, Braunschweig, Germany

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The filamentous hemagglutinin (FHA) of *Bordetella pertussis* was expressed in the attenuated *aroA* mutant of *Salmonella typhimurium*, SL3261, and in a strain of *Escherichia coli* harboring *Shigella flexneri* plasmid pWR110, which encodes bacterial invasiveness for epithelial cells. Expression of FHA in these strains did not interfere with their ability to invade Henle cells. Immunoglobulins A and G specific for FHA were detected in lung washes of mice following oral immunization with the live recombinant organisms; antibody levels were significantly higher than those in mice immunized with killed bacteria administered orally or intraperitoneally. Live oral vaccines carrying protective antigens of *B. pertussis* may be an important alternative to new-generation component vaccines against whooping cough.

Bordetella pertussis is the etiological agent of whooping cough, an infection of the upper and lower respiratory tracts. The disease is particularly severe in young children and may lead to neurological disorders and death (45). The incidence of whooping cough has been largely reduced by mass immunization with a heat-killed, whole-cell vaccine (11). However, concern about the infrequent but severe neurological side effects of this vaccine (6, 25) has led to a decreased acceptance rate (47) accompanied by an increase in disease incidence (33). New-generation vaccines composed of defined and nontoxic components are urgently needed.

Several virulence factors of *B. pertussis* have been considered for inclusion in defined acellular vaccines which would have reduced toxicity, and studies with animal models have highlighted pertussis toxoid and filamentous hemagglutinin (FHA) as prime candidates (20, 30). However, acellular vaccines composed of preparations of these two proteins exhibited lower efficacy than expected in human trials, despite their ability to induce a humoral response (1, 26, 43).

A number of vaccines that consist of live microorganisms which elicit better protection than killed organisms or cell extracts have been developed. More recently, several attenuated mutants of *Salmonella* spp. have been developed as delivery systems to stimulate mucosal immunity to cloned heterologous immunogens (19). These mutants are unable to multiply extensively or cause disease in the host but do establish a self-limiting, subclinical infection and can be detected in tissues such as the liver and spleen (5, 19). Mucosal, humoral, and cell-mediated immunity to antigens expressed by these strains can be induced by oral immunization (3, 9, 10, 19, 29, 35). Such delivery systems are, therefore, quite promising for vaccine development, and recently *Salmonella typhi* strains and capsule formulations suitable for oral administration have been accepted by the U.S. Food and Drug Administration for human use (22).

A related antigen delivery system is *Escherichia coli* K-12 containing the virulence plasmid pWR110 of *Shigella flexneri*, which specifies bacterial invasion of mammalian cells (16, 17, 23, 39, 40). This bacterium invades HeLa cells in tissue culture and produces a mild inflammatory reaction in the lamina propria in the rabbit ileal loop test (38). Monkeys orally immunized with *E. coli* K-12(pWR110) expressing *S. flexneri* 2a surface antigens were protected against oral challenge with virulent *S. flexneri* 2a bacteria (12).

Here we describe the oral immunization of mice with *Salmonella typhimurium aroA* and invasive *E. coli* hybrids expressing the 220-kDa FHA of *B. pertussis* and the induction of both systemic immunoglobulin G (IgG) and mucosal IgA anti-FHA antibodies.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work were *E. coli* EC538 (J. E. G. McCarthy), *S. typhimurium aroA* SL3261 (19), and *E. coli* K-12 strain 395-1 (38). The plasmids used in this work were pCG26 (Ap^r) (15), pWR110 (Km^r), and R64*drd*11 (Tc^r) (17, 38, 39).

E. coli and *S. typhimurium* strains were grown in Luria broth or on Luria agar plates (36). *B. pertussis* was grown on Bordet Gengou agar base (Difco) supplement with 1% glycerol and 15% (vol/vol) defibrinated horse blood or SS-X broth (42). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; tetracycline, 50 μg/ml; and kanamycin, 50 μg/ml. Broth cultures were aereated by shaking at 200 rpm in a New Brunswick Environmental Incubator Shaker.

Induction experiments. These experiments were per-

* Corresponding author.
† Present address: Institute of Microbiology, University of Genoa, Viale Benedetto XV, 10, 16132 Genoa, Italy.
‡ Present address: Department of Biology, University of Wollongong, Wollongong, NSW 2500, Australia.

formed as previously described (15). Protein concentrations of samples were determined by the Lowry procedure as modified by Sanderman and Strominger (37).

Tissue culture methods and in vitro determination of adhesiveness and invasiveness. The human intestinal epithelial cell line Henle 407 (ATCC CCL-6) was maintained in Dulbecco's modified Eagle medium (DME; GIBCO Laboratories, Eggenstein, Germany) with 10% fetal calf serum (FCS) (GIBCO)–5 mM glutamine (Flow Laboratories Inc., Mclean, Va.)–1 mM pyruvate (Flow) in an atmosphere containing 5% CO_2 at 37°C. Trypsinized cells were seeded at a concentration of approximately 2×10^5 cells per coverslip (15 by 15 mm) in tissue culture plates (12 by 4.5 cm^2; Flow). These were incubated for 18 to 24 h and washed three times in phosphate-buffered saline (PBS; NaCl [8.0 g $liter^{-1}$], KCl [2.0 g $liter^{-1}$], $Na_2HPO_4 \cdot 2H_2O$ [2.0 g $liter^{-1}$], KH_2PO_4 [2.0 g $liter^{-1}$]) (pH 7.4). For infection of monolayers, bacteria were harvested in the exponential growth phase by centrifugation, resuspended to a density of 10^8 CFU/ml in DME, overlaid onto the monolayer of cells on a coverslip, and incubated for 30 min at 37°C with 5% CO_2. Unattached and loosely attached bacteria were removed by washing the monolayers three times with PBS. For adhesiveness assays, the monolayers were then stained with Giemsa solution and examined by light microscopy, and the mean number of bacteria per Henle cell was calculated by averaging the number of bacteria adhering to 40 cells. For invasiveness assays, DME containing gentamicin (100 μg/ml) was then added; this concentration of gentamicin killed extracellular bacteria and thereby prevented any reinfection of cells. Monolayers were incubated for an additional 3 h and then washed three times with PBS. The number of invading bacteria was determined both by selectively staining the viable intracellular microorganisms by using the method described by Pruzanski et al. (32) and by lysing the eukaryotic cells with 1 ml of ice-cold water added to each well and then determining the viable count on Luria agar plates.

DNA manipulations. Plasmid DNA isolation and transformation were performed as described by Sambrook et al. (36). Conjugal transfer of pWR110 (Km^r) to *E. coli* EC538 was accomplished with the mobilizing helper plasmid R64*drd*11 (Tc^r), as previously described by Sansonetti et al. (38, 39).

Mouse immunization. Six- to seven-week-old female BALB/c mice were immunized and caged separately. Mice were immunized as shown in Table 1. Groups a to i were immunized with one dose on day 0 and boosted with an identical dose 24 days later. The animals were sacrificed 10 days after the booster, and the samples were collected. For groups j and k, only 1 dose was given, and the mice were sacrificed 24 days later. For peroral immunization, an overnight broth culture of bacteria was diluted 100-fold with Luria broth containing an appropriate antibiotic for selection and the fresh culture was incubated at 37°C until it reached early logarithmic phase. The cultures were centrifuged, and the bacteria were resuspended in PBS to an appropriate optical density to give the desired viable count as determined previously for each strain. Immediately before immunization, an equal volume of 3% sodium bicarbonate in PBS (pH 8.0) was added to the suspension. Mice that had been deprived of water for 6 to 8 h were then gently fed with 50 μl of the bacterial suspensions. For immunization with heat-killed bacteria, bacterial suspensions in PBS were treated at 60°C for 30 min. Sacrificed mice were exsanguinated by cutting the brachial artery, and the collected sera were separated and stored at −20°C. Lung washes were collected by pertracheal cannulation and gentle washing with 0.7 ml of ice-cold PBS containing 2 mM phenylmethylsulfonylfluoride as the protease inhibitor. About 0.5 ml of lung wash was recovered from each mouse, centrifuged at 4°C at 3,000 × *g* for 5 min to remove debris, and stored at −20°C.

TABLE 1. Mouse immunization protocols

Group[a]	Strain	No. of mice	Dose	Route	Viability
a	SL3261	6	10^9	Oral	Live
b	SL3261(pCG26)	6	10^9	Oral	Heat killed
c[b]	SL3261(pCG26)	5	10^9	Oral	Live
d	SL3261(pCG26)	6	10^6	i.p.	Live
e	EC538(pCG26)	6	10^9	Oral	Live
f[b]	EC538(pCG26, pWR110)	6	10^9	Oral	Live
g	*B. pertussis* Tohama	6	10^6	i.p.	Heat killed
h	SL3261	6	10^6	i.p.	Heat killed
i	SL3261(pCG26)	6	10^6	i.p.	Heat killed
j	SL3261(pCG26)	5	10^9	Oral	Live
k	EC538(pCG26, pWR110)	5	10^9	Oral	Live

[a] Groups a to i were immunized twice (days 0 and 24) and sacrificed 10 days after the booster; groups j and k were immunized once and sacrificed 24 days later.

[b] Experiments with these groups were repeated with independently prepared vaccines and the same number of mice (five and six, respectively). Results in Fig. 2 refer to averages from both experiments.

Immunological techniques. Monoclonal antibody P12H3 against FHA was kindly provided by C. Parker (13). For Western blot experiments, whole-cell extracts of bacteria were mixed with loading buffer (36) in a ratio of 1:1, and approximately 50 μg of protein was electrophoresed according to the procedure of Laemmli (21) by using a 3.85% acrylamide stacking gel and a 10% acrylamide separating gel. Blotting was carried out essentially as described by Burnette (4). Proteins were transferred to a nitrocellulose membrane with a semidry transfer cell by using 25 mM Tris–192 mM glycine–20% methanol, pH 8.3, as the transfer buffer and a 10% solution of 0.3% fat milk in PBS, pH 7.4, as the blocking reagent. The blocked membrane was incubated for 2 h with the first antibody (hybridoma P12H3 supernatants diluted 1:20 in PBS), washed three times with PBS, and incubated for 1 h with Bio-Rad horseradish peroxidase-conjugated goat anti-mouse IgG. Membranes were washed and developed with 4-chloro-1-naphthol as a substrate. Prestained molecular weight markers were purchased from Bio-Rad.

For the determination of subclass-specific antibodies against FHA in serum and lung washes, enzyme-linked immunosorbent assays (ELISA) were performed as follows. Nunc Maxisorp Immunomodule 96-well plates were coated with FHA purified from *B. pertussis* Tohama by the procedure of Sato et al. (41) and diluted in 0.1 M $NaHCO_3$ (pH 9.6; 60 ng in 50 μl per well; incubation at 4°C overnight). The wells were blocked with 100 μl of 10% FCS in PBS for 2 h at 37°C. Plates were subsequently washed three times with PBS, and 100 μl of serum samples diluted 1:25 or lung washes diluted 1:10 in 10% FCS in PBS was added to each well. After 60 min at 37°C, the plates were washed again and 100 μl of peroxidase-conjugated goat anti-mouse antibodies for IgG (Fc), IgM (Jackson Immunoresearch Laboratories), or IgA (Southern Biotechnology Associates, Inc.) heavy chains diluted 1:1,000 in 10% FCS in PBS was added to each well and incubated 1 h at 37°C. The plates were washed again and developed by the addition of 200 μl of activated sub-

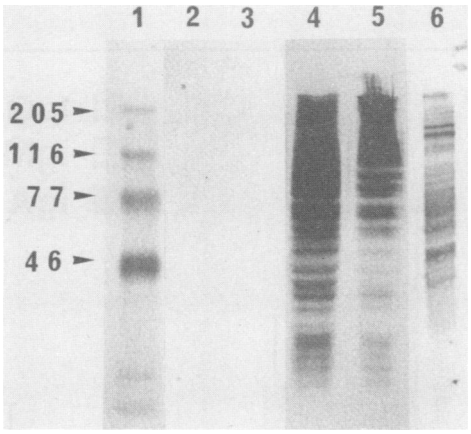

FIG. 1. Expression of nonfusion FHA in *E. coli* and *S. typhimurium aroA*. Total cell extracts were analyzed by Western blot by using P12H3 monoclonal antibody. Lanes: 1, molecular mass standards; 2 and 4, *S. typhimurium aroA* SL3261 containing pCG26; 3 and 5, *E. coli* EC538 containing pCG26 and pWR110; 6, *B. pertussis* Tohama. Expression was not induced by growth at 30°C (lanes 2 and 3) or induced for 3 h at 37°C (lanes 4 and 5). The molecular masses of the standards in kilodaltons are indicated.

strate solution (0.025 M citric acid, 0.05 M Na_2HPO_4, 0.015% H_2O_2, 0.3 g of *o*-phenyldiamine dihydrochloride $liter^{-1}$) per well. After 30 min at room temperature, the reaction was stopped by the addition of 50 μl of 0.25 M H_2SO_4 and the A_{490} was determined with a Bio-Rad 3550 microplate reader. All samples were processed simultaneously on the same day, normal nonimmunized mouse serum or lung wash was used as the blank for the ELISA readings, and each mouse serum or lung wash was individually assayed; standard deviations represent variations between individual mouse samples in each group.

RESULTS

Expression of recombinant nonfusion FHA in *S. typhimurium* and *E. coli*. The construction of recombinant *S. typhimurium aroA* SL3261(pCG26) and *E. coli* EC538(pCG26) strains was described previously (15). In these strains, nonfusion recombinant FHA (200 to 220 kDa) is either produced as a soluble cytoplasmic protein (EC538) or deposited in inclusion bodies (SL3261). The wild-type and the recombinant protein have similar migration patterns on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The pWR110 (Km^r) 140-MDa virulence plasmid encoding the invasive phenotype of *S. flexneri* was mobilized into EC538(pCG26) (Ap^r) by using the helper plasmid R64*drd*11 (Tc^r) and selecting for Ap^r Km^r transconjugants to produce EC538(pCG26, pWR110). In both recombinant strains, SL3261(pCG26) and EC538(pCG26, pWR110), the FHA gene is under the control of the thermoregulated tandem p_R and p_L promoters and the translation initiation region of the *E. coli atpE* gene. The first 15 codons of the FHA gene had been engineered to reduce mRNA secondary structure in the NH_2-terminal coding region. Expression is repressed at 30°C and induced at temperatures above 34°C. Western blot analysis (Fig. 1) demonstrated that in both SL3261 and EC538(pWR110) carrying pCG26, FHA is efficiently expressed at 37°C (lanes 4 and 5) and efficiently repressed at 30°C (lanes 2 and 3). Expression in the recombinants was higher than in wild-type *B. pertussis* Tohama (lane 6); extensive degradation was observed in all strains, although this may not be important for vaccine purposes. The presence of pWR110 did not significantly interfere with FHA expression. Cultures of SL3261(pCG26) and EC538(pCG26, pWR110) were grown overnight in Luria broth without antibiotics, and dilutions were spread onto Luria agar plates with and without antibiotics. The presence of antibiotic had no significant effect on the number of CFU obtained (data not shown), thus demonstrating reasonable stability of the hybrid plasmid in these host bacteria.

TABLE 2. In vitro adhesiveness and invasiveness of strains used in the vaccination protocols

Strain	Adhesiveness[a]	Invasiveness	
		% Infected cells[b]	CFU[c]
SL3261	13.3 ± 6.3	11	1.5×10^3
SL3261(pCG26)	12.1 ± 5	12	1.2×10^3
EC538(pCG26)	2.8 ± 1.8	2	<25[d]
EC538(pCG26, pWR110)	63.8 ± 9.9	98	7×10^4
E. coli K-12 395-1 (pWR110)	58.3 ± 10.7	95	5×10^4
EC538	1.7 ± 1.8	3	<25[d]

[a] Mean number of attached bacteria per Henle cell ± the standard deviation.
[b] Percentage of Henle cells invaded by at least five bacteria.
[c] CFU of viable intracellular bacteria per coverslip.
[d] Limit of detection.

In vitro adhesiveness and invasiveness of strains used in the vaccination protocols. The results presented in Table 2 show that the presence of hybrid plasmid pCG26 did not alter the adhesive or invasive properties of the carrier strains. *E. coli* harboring pWR110 adhered more efficiently to Henle cells than did the *Salmonella* strain, which in turn adhered more efficiently than *E. coli* lacking pWR110. The two methods used to assess invasiveness gave comparable results. No viable bacteria were isolated from invasion assays with *E. coli* lacking pWR110, and the few Henle cells observed microscopically to contain such bacteria may be artifacts. *E. coli* bacteria containing plasmid pWR110 were considerably more invasive than *Salmonella* bacteria. This is probably due at least in part to the inability of *S. typhimurium* to replicate in vivo as a result of its *aroA* mutation. However, the invasiveness of *S. typhimurium* was considerably greater when centrifugation was used to facilitate contact between bacteria and host cells (results not shown). The relevance of this to the in vivo situation is not, however, clear.

Antibody responses specific for *B. pertussis* FHA in vaccinated mice. Both FHA delivery systems elicited specific serum and mucosal immune responses (Fig. 2). Some correlation between the ability of carrier strains to invade Henle cells in vitro and their ability to stimulate antibody production in the lungs was observed. Higher immune responses from live vaccines are evident from a comparison of the immune responses induced by intraperitoneal (i.p.) immunization (compare group d with groups g and i in Table 1 and Fig. 2) and peroral immunization (compare groups b and c in Table 1 and Fig. 2). Moreover, IgA could be detected in the lungs only when the peroral route was used for delivering *S.*

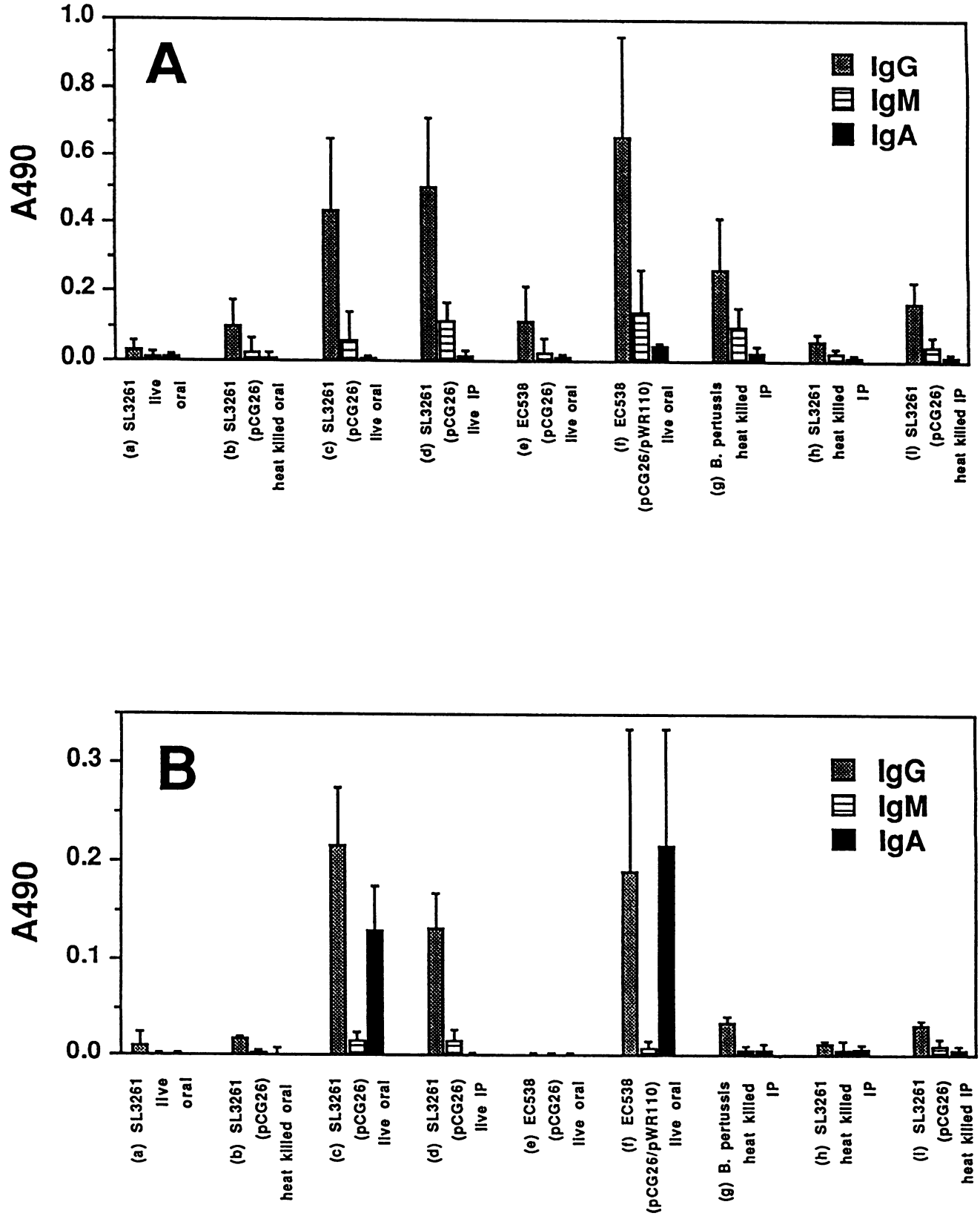

FIG. 2. Levels of specific antibodies in sera (A) and lungs (B) after immunization of mice. Standard deviations are indicated by vertical lines. IP, intraperitoneal.

typhimurium aroA or invasive *E. coli* live vaccines (compare groups c and f with groups b and d in Table 1 and Fig. 2B). After i.p. immunization with live SL3261(pCG26), FHA-specific IgG, but no IgA, was detected in serum and lung washes (IgG can diffuse from blood to the lower respiratory tract). The serum or lung wash samples in which specific immunoglobulins could be detected by ELISA also reacted with purified FHA in Western blots (data not shown).

The invasion plasmid pWR110 was essential for antibody induction (compare groups e and f in Fig. 2). Two doses of peroral immunization were necessary for the induction of good antibody titers: only one dose of either SL3261(pCG26) or EC538(pCG26, pWR110) (groups j and k) resulted in IgG serum ELISA readings of 0.15 for group j and 0.10 for group k and IgA lung wash readings of 0.03 for both j and k.

Significant specific IgM was not detected in lung washes after any immunization, but very low levels could be detected in serum after either oral immunization with SL3261(pCG26) or EC538(pCG26, pWR110) or i.p. immunization with *B. pertussis*.

DISCUSSION

Whooping cough is a disease with a respiratory tract portal of entry. To prevent infection, it is desirable to develop vaccines which stimulate not only specific serum antibodies but also specific IgA in the respiratory tract. Such secretory antibodies may interfere with the early events of bacterial attachment and colonization. The prevention of infection as well as disease is important if vaccines are to facilitate eradication of a disease for which there is only a human reservoir. From serological studies in patients with pertussis (44), increases in IgG and IgA against different virulence factors of *B. pertussis* were observed, whereas whole-cell-vaccinated individuals gave very weak, if any, increases in IgA titers.

The presence of specialized cells associated with the uptake of macromolecules in the gut (28) and the mucosal immunological network with subpopulations of lymphoid cells that can migrate from the intestine to other distant mucosae (e.g., the respiratory tract) (2, 24, 34), together with the experimental evidence of immune responses in serum and salivary glands after peroral immunization (7), indicate that live peroral vaccines may be good candidates for whooping cough. Our data clearly support the usefulness of this approach. Oral immunization of mice with live *S. typhimurium aroA* and invasive *E. coli* expressing *B. pertussis* FHA induced both serum IgG and secretory IgA antibody responses in the lungs.

Delivery systems based on *E. coli*(pWR110) offer more flexibility than *Salmonella* systems. The genetics of *S. typhimurium* is not as well developed as that of *E. coli*, and it is easier to optimize expression of recombinant antigens in *E. coli* and then transfer in the necessary invasion genes. Although we were fortunate in obtaining good expression of FHA in the one *Salmonella* strain selected, we found expression in *E. coli* to be highly strain dependent (15). Obviously, plasmids such as pWR110 which specify antibiotic resistance are unacceptable in human vaccines, but the existence of versatile transposon cloning vectors lacking antibiotic resistance markers that can stably integrate cloned genes into host chromosomes (18) will enable the cloning of invasion genes (23) and their insertion into the chromosomes of selected antigen carrier strains. The stability of immunogen-coding sequences and the absence of antibiotic resistance selection markers are crucial features of live vaccines. To achieve these goals, we are currently working on transposon-mediated chromosomal integration (18) of the FHA gene and the necessary expression signals needed to achieve high-level in vivo expression of FHA, by using an approach that previously gave good expression of the pertussis toxin operon in *Bordetella bronchiseptica* (46).

Recently, Parker and coworkers (27, 31) orally immunized mice with *Salmonella* bacteria expressing truncated FHA by using a host-vector system which causes release of the intracellular antigen and prevents loss of hybrid plasmids in vivo. Although this group was able to detect FHA-specific IgG in serum, they failed to detect specific IgA in lung washes. The strong mucosal immune responses measured in our experiments may be due either to a higher level of expression of FHA in pCG26-carrying bacteria or to the particular delivery strains used in this study. In addition, pCG26 specifies the entire 220-kDa protein, and it is possible that the native protein is more immunogenic than the truncated forms expressed in previous studies (27, 31).

The role of cell-mediated immunity in protection against *B. pertussis* is not known, although a cell-mediated response to several *B. pertussis* antigens, including FHA, has been demonstrated in convalescent patients (8, 14). As *Salmonella*-expressing heterologous antigens are known to induce cell-mediated immunity (3, 35), it will be of interest to determine whether the delivery systems used in this study induce cell-mediated mucosal responses to FHA in addition to an antibody response.

It is not known whether vaccines based solely on FHA can offer sufficient protection in humans against *B. pertussis* infection (26, 43, 44). However, the potential of these carrier systems to stimulate a strong mucosal immune response to *B. pertussis* antigens that we demonstrate here will encourage the construction of carrier strains expressing more than one *B. pertussis* antigen. The recent successful oral immunization against tetanus in mice by using the SL3261 carrier strain (10) supports this thesis and opens up new perspectives for the development of oral attenuated multivalent vaccines that could replace the old DTP vaccine in vaccination regimens.

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